THE EQUILIBRIUM BETWEEN HEMOGLOBIN AND OXYGEN IN THE BLOOD OF CERTAIN FISHES

ARDA ALDEN GREEN AND RAYMOND W. ROOT
THE WOODS HOLE OCEANOGRAPHIC INSTITUTION [1]

In studies made on the hemoglobin of certain teleost fishes Root (1931) found that carbon dioxide had a remarkable effect on the ability of the blood to combine with oxygen. As in the case of mammalian blood, the addition of acid decreases the amount of oxygen combined with the hemoglobin at any given oxygen tension, but in these fish bloods the addition of acid decreases also the total amount of oxygen that can be combined even when the oxygen tension is increased to that of air. As a consequence the suggestion was made that the prosthetic groups in the hemoglobin molecule were differentially affected by the presence of acid, some being inactivated entirely so that they no longer combined with oxygen. Since confirmation of this suggestion promised to throw new light on the theory of the equilibrium between oxygen and hemoglobin, the phenomenon has been examined in greater detail.

In the present study we have examined the blood of the tautog, *Tautoga onitis* Linnæus, the goosefish, *Lophius piscatorius* Linnæus, and the toadfish, *Opsanus tau* (Linnæus). We have confirmed the observation of Root and shown that the amount of oxygen combined is decreased in the presence of acid even though the oxygen tension is raised to one atmosphere. In addition, the oxygen dissociation curves have been studied throughout the range of hydrogen ion concentration in which the phenomenon is described. These curves are remarkable as to shape, especially those of the toadfish, which have a distinct undulatory character. Another characteristic is a change in the shape of the curves with a change in pH. In alkaline solutions the curves for the goosefish and for the tautog are sigmoid whereas in more acid solutions the curves are rectangular hyperbolæ. Thus in these teleost fishes we find certain unique characteristics exhibited by the oxygen dissociation curves. Such peculiarities must be accounted for by any acceptable general theory of the union of oxygen with hemoglobins.

METHODS

The blood of the goosefish was obtained from the bulbus or sinus venosus, and was removed from the animal shortly after it was taken

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from the commercial fish traps. On account of the low hemoglobin concentration of the blood of this fish it was customary to centrifuge the blood and draw off plasma equal to about one-third the total volume, before making analyses. The other fishes were maintained at the laboratory in tanks, and were bled from the gills by means of a hypodermic syringe. Lithium oxalate was used as an anticoagulant. Samples of blood were necessarily combined in any given species of fish and the reserve supply kept on ice. In most cases the blood was used within 12 hours. In the case of the goosefish it was possible to keep a sample of blood several days without the appearance of methemoglobin.

The usual methods of obtaining oxygen dissociation curves were employed as described by Dill (1928). Carbon dioxide and oxygen analyses on the blood were done simultaneously as described by Root (1931). The analysis of gas mixtures containing over 30 per cent carbon dioxide or oxygen was made possible by a special modification of the Haldane apparatus. Gas pressures in the tonometers were calculated by use of the formula of Bock, Field, and Adair (1924). Samples of blood were equilibrated at a temperature of 25° C. for 15 minutes in a water bath. Nitrogen was used as the inactive gas in the tonometers. It is essential to point out that but one tonometer was equilibrated at a time and analyses followed directly after equilibration. This procedure is quite necessary in fish blood, since it possesses active, nucleated erythrocytes (Dill, Edwards, and Florkin, 1932). Duplicate analyses, both of the gases in the blood and in the tonometer, were made for all established points.

Since the oxygen combined by these bloods is diminished in the presence of acid, the oxygen capacities were always determined on blood to which a small amount of powdered sodium bicarbonate had been added. This blood was then equilibrated either in air or in oxygen and the figure obtained after dissolved oxygen was subtracted was taken to be the real oxygen capacity of the hemoglobin.

A few of the more acid samples turned brownish in color and the formation of methemoglobin was suspected. In such cases the oxygen capacity of a duplicate sample of the blood was determined directly after each equilibration by using sodium bicarbonate to bring the blood once more to an alkaline reaction and re-equilibrating with air. Whenever the hemoglobin had been irreversibly inactivated the percentage saturation was calculated by using the oxygen capacity as determined after equilibration rather than the original value.

All the data expressing the quantity of oxygen in blood were corrected for dissolved oxygen. In the case of toadfish and goosefish

the solubility coefficient for oxygen was experimentally established as 2.70 vols. per cent for 760 mm. O_2 at 25° C. This figure was assumed to hold in the case of the tautog.

In studies of the effect of acid on the ability of blood to combine with oxygen, samples of blood were equilibrated in oxygen or air to which had been added various amounts of carbon dioxide. When acidities were desired greater than could be obtained in this way a few drops of 4 per cent lactic acid were added.

The pH of fish blood is not easy to control and great difficulties were experienced in trying to establish dissociation curves at constant pH. On account of the great change in acidity of the hemoglobin with degree of oxygenation the use of a constant carbon dioxide tension does not lead to a constant pH at all oxygen tensions. This difficulty was overcome by grading the amount of carbon dioxide added and, in some cases, by adding a small amount of sodium bicarbonate. In this way it was possible to obtain points calculated to have approximately the same pH values at all degrees of oxygenation. The pH of the blood was calculated from gasometric data, using the Henderson-Hasselbalch equation and assuming pK 6.22 for whole blood at 25° C. Dissolved carbon dioxide was calculated by using Bohr's solubility coefficient slightly modified in a manner prescribed by Peters, Bulger, and Eisenman (1924) to allow for differences in corpuscular volume. The final factors used to calculate the volume percentage of dissolved CO_2 were 0.102 pCO_2 for toadfish, and 0.1 pCO_2 for the other fish bloods. It must be remembered that the electrolyte equilibrium between cells and plasma is completely ignored and the pH is calculated from determinations of the carbon dioxide equilibrium of whole blood rather than that of either plasma or cells.

The Apparent Loss of Oxygen Capacity in Acid Solutions

Root (1931) found that the amount of oxygen combined with certain fish bloods at a pressure of oxygen equivalent to that of air depended upon the acidity of the blood. The addition of very small amounts of carbon dioxide (even 5 or 10 mm.) or of lactic acid, appreciably decreased the oxygen bound. This effect is reversible, for if the blood equilibrated under acid conditions is again made alkaline it regains its original combining capacity.

It seemed to us that this phenomenon might be merely a pronounced "Bohr" effect (Bohr, Hasselbalch, and Krogh, 1904) in which the acid altered the oxygen dissociation constant to such an extent that the oxygen tension of air was insufficient to completely saturate the hemoglobin. If higher oxygen tensions had been used, saturation

might have been complete and the oxygen capacity undiminished. To test this possibility fish blood was equilibrated with oxygen containing the requisite quantity of carbon dioxide. Since the total pressure was about one atmosphere, the oxygen tension of these gas mixtures decreased with increasing carbon dioxide concentration. For this reason lactic acid was added on some of the more acid samples to enable a relatively high acidity to be reached without too greatly diluting the oxygen with carbon dioxide.

The results upon the blood of the goosefish, the tautog, and the toadfish in both air and oxygen at 25° C. are represented in Fig. 1.

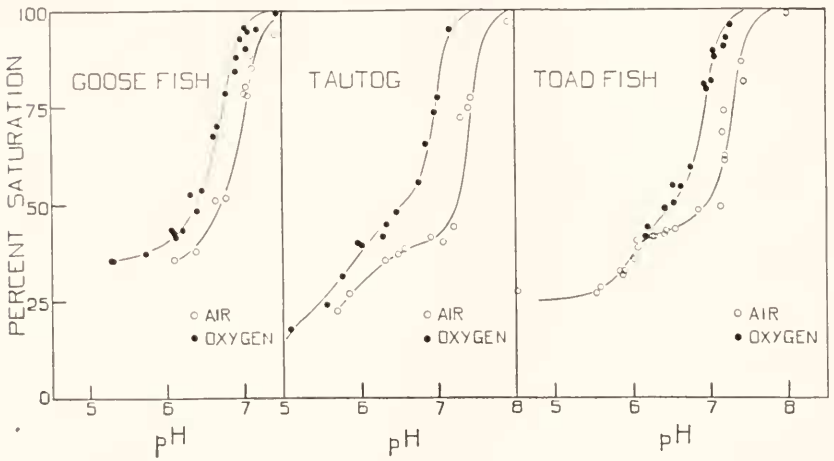

Fig. 1. The oxygen combined in fish bloods equilibrated with varying tensions of carbon dioxide and oxygen or of carbon dioxide and an oxygen tension approximating that of air.

The oxygen tension of the more acid points in the curves of the toadfish is about 500 mm., of the tautog 650 mm., and of the goosefish 350 mm. Except in the acid ranges of the toadfish blood, the samples equilibrated with oxygen combined with more oxygen than those treated with air. But it still remains a question as to whether the oxygen dissociation curves have shifted or whether the amount of oxygen which can be combined has been decreased. It seemed desirable, consequently, to determine the entire oxygen dissociation curves at various hydrogen ion concentrations in the hope that their analysis would enable us to determine the relative part played by these two phenomena.

Equations for Oxygen-Hemoglobin Equilibrium at Constant pH

There have been three types of equations describing oxygen dissociation which have proved useful for our purpose. The first of these

is that of Hill (1910). The fraction of hemoglobin combined with oxygen, in the following manner:

$$Y = \frac{Kx^n}{1 + Kx^n}, \tag{1}$$

where x is the oxygen concentration expressed as pressure in mm. Hg. Equation (1) may be derived from the mass law on the assumption that n is the number of molecules of oxygen uniting with each molecule of hemoglobin.

According to Adair's hypothesis, each molecule of hemoglobin combines with four molecules of oxygen and the molecule $Hb(O_2)_4$ is built up and broken down in stages, thus:

$$Y = \frac{0.25K_1(O_2) + 0.5K_2(O_2)^2 + 0.75K_3(O_2)^3 + K_4(O_2)^4}{1 + K_1(O_2) + K_2(O_2)^2 + K_3(O_2)^3 + K_4(O_2)^4}, \tag{2}$$

in which K_1, K_2, K_3, and K_4 are products of mass law equilibrium constants. This equation has been successfully used to describe the curves for man (Adair, 1925), the horse (Ferry and Green, 1929), and the sheep (Ferry and Pappenheimer, 1929).

Another expression has been suggested by Redfield (Ferry and Green, 1929, p. 194) and has been found useful in describing the oxygen dissociation curves of a number of bloods containing hemocyanin which have a distinct undulatory character (Redfield, 1933). It may be assumed that the respiratory protein consists of two or more components, each of which reacts with oxygen independently of the others and reaches an equilibrium described by a distinct oxygen dissociation curve. Further, each component reacts with oxygen in accordance with Hill's equation (equation 1) but the components are each characterized by a different value of n. If the oxygen dissociation constants of the forms characterized by values of n of 1.0, 2.0, 3.0, 4.0, etc. are designated by K_1, K_2, K_3, K_4, etc., and the fraction of the total oxygen bound by each of these forms as α_1, α_2, α_3, α_4, etc., the fraction of the total respiratory protein present in the oxygenated condition, Y, is given by the equations:

$$Y = \frac{\alpha_1 K_1 x^1}{1 + K_1 x^1} + \frac{\alpha_2 K_2 x^2}{1 + K_2 x^2} + \frac{\alpha_3 K_3 x^3}{1 + K_3 x^3} + \frac{\alpha_4 K_4 x^4}{1 + K_4 x^4} \cdots$$

In applying this expression to the fish bloods which we have studied, it is not necessary to employ more than the first two terms so that the expression becomes

$$Y = \alpha_1 \frac{K_1(O_2)}{1 + K_1(O_2)} + \alpha_2 \frac{K_2(O_2)^2}{1 + K_2(O_2)^2}. \tag{3}$$

TABLE I

Oxygen Dissociation of Toadfish Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
17	8.76	1.35	1.32	6.98	18.9	38.8	67.3	7.42
	19.4	2.57	2.50		35.8	36.3	64.7	7.44
	25.3	2.96	2.87		41.1	34.7	65.8	7.47
	44.25	3.71	3.55		50.9	36.7	67.0	7.45
	69.8	4.47	4.22		60.5	34.3	62.9	7.45
	80.0	4.62	4.34		62.2	36.1	65.1	7.44
	90.9	5.11	4.79		68.6	34.0	63.0	7.45
	107.0	5.26	4.88		70.0	35.1	64.2	7.45
	115.0	5.28	4.87		69.8	36.4	63.2	7.43
	117.5	5.30	4.88		69.9	34.9	64.0	7.45
	134.2	5.81	5.33		76.4	34.75	63.2	7.45
	151.0	6.21	5.68		81.4	33.85	61.55	7.44
	172.0	6.56	5.95		85.3	36.3	61.4	7.41
	191.0	6.72	6.04		86.6	34.1	63.5	7.44
	261.5	7.27	6.34		90.8	35.4	58.2	7.40
19 †	2.69	0.47	0.46	7.21	6.39	46.3	48.2	7.18
	14.7	1.95	1.90		26.3	45.2	47.5	7.19
	26.9	2.81	2.72		37.7	42.5	47.4	7.22
	53.8	3.41	3.22		44.7	41.4	46.35	7.22
	80.0	3.77	3.49		48.4	41.3	46.85	7.22
	102.0	4.16	3.80		52.7	42.0	46.0	7.21
	131.4	4.54	4.07		56.5	41.0	46.2	7.21
	147.0	4.94	4.42		61.3	43.0	45.1	7.19
	163.4	5.24	4.66		65.2	38.9	44.7	7.23
	209.5	5.79	5.05		70.0	43.8	44.1	7.17
	247.0	6.31	5.44		75.5	40.75	42.8	7.19
	322.0	6.73	5.59		78.2	40.25	43.0	7.20
	410.0	7.44	5.99		83.8	39.5	41.5	7.19
	512.0	8.01	6.19		86.5	39.8	41.8	7.19
	723.0	9.10	6.53		90.6	39.2	41.2	7.19
18	4.23	0.71	0.69	7.15	9.70	35.6	23.6	6.96
	16.7	1.97	1.91		26.7	35.2	23.3	6.96
	25.1	2.53	2.44		34.1	34.9	22.5	6.95
	53.2	3.50	3.31		45.3	34.2	22.6	6.96
	107.1	3.89	3.51		49.2	34.4	23.05	6.96
	137.7	3.98	3.49		48.8	45.7	24.7	6.83
	150.0	4.11	3.58		50.0	32.0	22.2	6.98
	225.0	4.66	3.86		53.0	31.6	22.2	6.99
	291.0	5.18	4.15		58.0	34.2	24.8	7.01
	352.0	5.59	4.34		61.0	34.2	22.3	6.95
	388.0	6.06	4.68		65.5	33.6	23.6	6.99
	461.0	6.54	4.90		68.6	34.4	23.5	6.98
	500.0	6.93	5.16		72.1	33.3	21.4	6.94
	729.0	8.37	5.68		79.5	34.6	22.4	6.96

TABLE I—*Continued*

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
6	4.23	0.65	0.64	6.04	10.6	51.85	28.3	6.86
	6.96	0.86	0.84		13.9	47.75	27.2	6.88
	16.65	1.70	1.65		27.2	50.7	27.2	6.85
	33.9	2.30	2.19		36.2	59.6	29.0	6.80
	69.9	2.76	2.53		41.8	53.9	26.9	6.81
	96.6	3.05	2.73		45.2	53.9	32.5	6.92
9	200.	3.62	2.91	6.20	47.0	46.3	26.2	6.89
	285.	3.98	2.97		48.0	47.7	30.3	6.94
	331.	4.30	3.13		50.5	46.6	25.9	6.88
	345.	4.60	3.38		54.5	47.3	25.2	6.86
	402.	5.02	3.60		58.0	44.5	22.7	6.83
	481.	5.32	3.61		58.2	46.6	25.8	6.87
	580.	5.59	3.54		57.1	45.5	26.9	6.91
	649.	5.94	3.64		58.7	46.1	24.5	6.86
5	4.90	0.61	0.61	6.16	9.9	120.2	39.55	6.57
	8.17	0.89	0.88		14.3	119.4	39.65	6.57
	12.74	1.18	1.16		18.8	110.8	38.25	6.60
	20.82	1.65	1.57		25.5	129.5	40.55	6.54
	36.14	2.05	1.90		30.8	123.0	40.00	6.56
	69.2	2.56	2.33		37.8	125.5	41.05	6.56
14	6.05	0.665	0.64	6.06	10.7	100.7	18.68	6.13
	13.10	1.015	0.97		16.0	100.2	18.40	6.12
	23.45	1.38	1.30		21.5	100.5	18.33	6.12
	33.7	1.63	1.51		24.9	97.2	18.10	6.13
	40.8	1.90	1.76		29.0	98.1	17.80	6.11
	48.7	2.02	1.85		30.5	97.7	17.48	6.10
	58.6	2.21	2.00		33.0	98.8	17.95	6.11
	91.0	2.44	2.12		35.1	97.4	18.00	6.13
	140.6	2.84	2.34		38.6	105.3	18.17	6.06

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

In order to take account of the apparent inability of all of the hemoglobin to combine with oxygen, which characterizes the more acid samples, it is necessary to introduce a term α_0, equal to the fraction of total hemoglobin incapable of combining with oxygen. It follows that

$$\alpha_0 + \alpha_1 + \alpha_2 = 1.$$

THE OXYGEN DISSOCIATION CURVES OF TOADFISH BLOOD

The results of the experiments on the blood of the toadfish are given in Table I and the oxygen dissociation curves at constant pH are shown in Fig. 2.

These curves may be observed to have certain interesting characteristics regardless of the theory or of the equations used to describe the oxygen-hemoglobin equilibrium. The first of these is a difference in the shape of the curves at different hydrogen ion concentrations. In mammalian blood, on the contrary, curves at constant carbon dioxide tension or at constant pH are apparently of one family having the same shape.

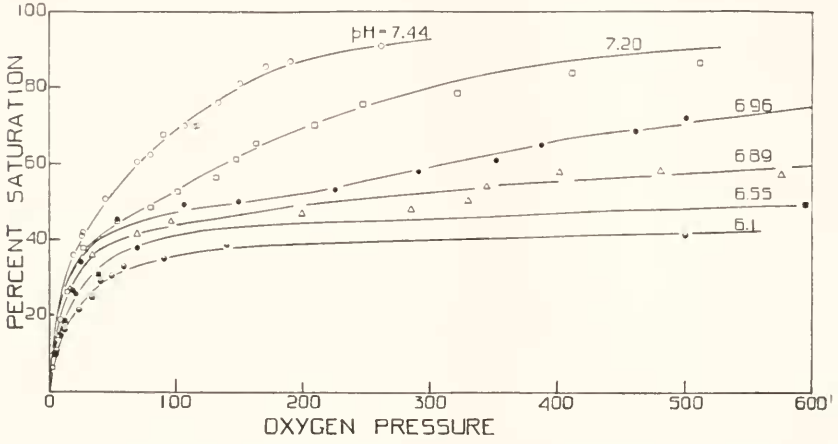

FIG. 2. Oxygen dissociation curves at constant pH of the blood of the toadfish. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_3	K_1	$K_2 \times 10^4$	$1/K_1$	$1/K_2$
7.44	.5	.5	0	.11	.826	9	110^2
7.20	.5	.5	0	.10	.186	10	232^2
6.96	.5	.48	.02	.10	.033	10	548^2
6.89	.5	.2	.3	.071	.033	14	548^2
6.55	.5	0	.5	.0475		21	
6.1	.43	0	.57	.0475		21	

The data may be equally well described by equation (2) when the following values for the constants are used:

pH	K_1	$K_2 \times 10^2$	$K_3 \times 10^4$	$K_4 \times 10^6$
7.44	.2	1.0	.01	1.0
7.20	.1	.6	.04	.10
6.96	.1	.5	.001	.015
6.89	.1	.4	.001	.004
6.55	.1	.2	.001	.0001

A second effect of pH on these curves is a shift to the right with increasing acidity, a phenomenon well established for various mammalian bloods. On the other hand, the change in the position of the curve with change in pH is almost insignificant in the blood of the elasmobranch, *Raia ocellata* (Dill, Edwards, and Florkin, 1932). In these marine teleosts, and especially in the toadfish, the effect is very marked. As a result the curves determined at pH 7.2, 6.96, and 6.89 are not completely saturated even in the presence of 500 mm. O_2 and the shape of the curves indicates that more oxygen would be combined at higher oxygen tensions.

This is an explanation of a part of the decrease in oxygen capacity described by Root (1931). It should be noted, however, that the curves determined at pH 6.55 and 6.1 are practically flat at tensions of oxygen above 150 mm. which suggests that saturation is complete but that a part of the hemoglobin is incapable of combining with oxygen.

The next important feature of the curves is their peculiar shape. Some of them are not simple hyperbolic or sigmoid curves but are undulatory in character, having two regions concave to the abscissa. Similar types of curves have been found for the blood of some birds (Wastl and Leiner, 1931) and of certain animals containing hemocyanin (Redfield, 1933). The break between the two regions of the curves occurs at about 50 per cent saturation, and the more acid curves, in which the combined oxygen has become constant, do not rise above 50 per cent saturation. This characteristic of the curves suggests that the oxygenation of the hemoglobin consists of two steps, or that there are two components to the curves representing the behavior of different fractions of the pigment present in equal quantities. Theoretical treatment of these possibilities is provided by the equations given above.

The equation (1) developed by Hill applies only to simple curves, hyperbolic or sigmoid in shape, and thus could be used to describe only the most acid curves and these only if the decrease in oxygen capacity is assumed to be real so that higher oxygen tensions would not increase the oxygen bound.

Although Adair's theory in which the hemoglobin combines with oxygen in steps was derived for sigmoid curves, equation (2) may be used to describe the oxygen dissociation curves of the toadfish. By making K_3 and K_4 sufficiently small, the terms in which they appear become ineffective at the oxygen tensions used and the curve becomes flat at one half saturation, as in the experiment at pH 6.55. According to this treatment the decrease in oxygen capacity is only apparent and

the effect really one on the magnitude of the constants and thus a "Bohr" effect. However, the curve at pH 6.1 is indubitably flat over a long range at less than 50 per cent saturation, that is, at 43 per cent, and it is also apparent from Fig. 1 that a further increase in acidity would further decrease the oxygen bound. Such curves can only be derived from the Adair equation by assuming a true loss in oxygen capacity, thus necessitating the introduction of a new constant to describe this loss.

The curves may be described equally well in terms of Redfield's equation which employs fewer constants. The smooth curves in Fig. 2 are drawn according to equation (3). When the curves have not flattened out at one atmosphere of oxygen, the fraction of hemoglobin incapable of combining with oxygen must be calculated from the shape of the dissociation curves at lower tensions and this unfortunately gives an additional degree of freedom in analyzing some of the curves.

The difference between the values for K_1 and K_2 gives the undulatory character to the curves. In some cases the hemoglobin combining with oxygen according to the first constant is almost completely saturated before the second inflection begins. By taking α_1 and α_2 each = 0.50, which implies that the amount of hemoglobin acting as though it combined with one molecule of oxygen at a time exactly equals that combining with two molecules, the flat places in the curves or the points where there is a change of inflection occur at about one half saturation.

The decrease in oxygen capacity takes place first at the expense of the component behaving as though $n = 2$. The value of α_1, the amount of hemoglobin behaving according to the first term of the equation, remains constant at 0.50 throughout the pH range until the amount of hemoglobin capable of combining with oxygen has been reduced to 50 per cent of the whole. Thus, the curve at pH 6.55, in which the capacity is 50 per cent of the total oxygen-combining power, is a rectangular hyperbola. Further addition of acid reduces the capacity but the curve is still of the same shape and has the same value for the constant K_1. This implies that the effect described by Root comprises a real inactivation of the hemoglobin and is due only in part to a greatly exaggerated "Bohr" effect.

All three treatments have certain common implications. The description of the curves in accordance with either equation (1), (2), or (3) requires the assumption that a portion of the hemoglobin has lost its ability to combine with oxygen in the case of the most acid solution. In the use of equation (3) it is implied that the prosthetic

groups responsible for the two components of the curves are divided in equal amounts. This condition could be met if there were two forms of hemoglobin present, but the probability of two independent substances existing in such exact proportion is rather small. It is much simpler to postulate the existence of a number of oxygen-combining groups on one molecule, one half of which behave in one way and one half in the other. Since one half of the group behave as though they combined with oxygen in pairs, at least four prosthetic groups must be attributed to the molecule. In this regard the implications regarding the structure of the hemoglobin molecule are the same as those postulated by Adair in deriving equation (2).

The Oxygen Dissociation Curves of the Blood of the Tautog and of the Goosefish

The blood of the tautog and of the goosefish may be considered together since they exhibit similar characteristics. The data are presented in Tables II and III.

The most important characteristic of these curves is a change in the shape of the curve with a change in pH. Equation (1) may be transformed to the logarithmic form:

$$\text{Log}\frac{\text{HbO}_2}{\text{Hb}} = \log K + n \log \text{pO}_2.$$

When log HbO_2/Hb is plotted against log pO_2, the resulting curve is a straight line, the slope of which is equal to n. The data for these two fish bloods have been calculated in this manner and the results plotted in Fig. 3. In both cases the most alkaline curves are straight lines and the slope is 2.0, that is, Hill's equation is applicable and n is 2.0. In both cases, also, the most acid curves are straight lines but here n is 1.0 and consequently the oxygen dissociation curves are rectangular hyperbolæ.

In both these bloods the oxygen-combining power is decreased in the more acid solutions. In the tautog the curve has changed to a rectangular hyperbola ($n = 1.0$) at pH 7.2. At this reaction the oxygen capacity is not appreciably lowered, so that the change in shape is independent of this phenomenon. In the goosefish the decrease in oxygen capacity begins to appear before the curves have assumed a shape characterized by $n = 1$, and in applying Hill's equation it is necessary to make allowances for the decreased oxygen capacity of the blood, as was done in the case of toadfish blood (p. 391). At pH 6.84, 6.80, and 6.1 the oxygen capacities are assumed to be 88, 80, and 54 per cent respectively, of those obtaining in alkaline solutions.

Table II

Oxygen Dissociation of Goosefish Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
3	2.77	2.80	2.79	8.40	33.2	2.53	24.1	8.20
	7.08	5.70	5.68		67.5	3.16	25.2	8.11
	10.4	7.02	6.98		83.0	2.14	22.9	8.23
	15.94	7.90	7.84		93.3	2.41	23.2	8.20
	66.9	8.74	8.50		101.0	2.76	24.2	8.15
4 †	5.26	5.07	5.05	8.59	58.8	6.36	51.3	8.12
	6.79	5.64	5.62		65.5	8.54	56.2	8.03
	7.74	6.42	6.39		74.4	6.17	50.4	8.13
	7.89	6.35	6.32		73.6	7.23	54.3	8.09
	12.33	7.50	7.46		86.8	7.20	53.9	8.09
	14.58	7.96	7.91		92.2	6.29	52.0	8.15
	25.1	8.32	8.23		95.8	6.22	51.2	8.13
3	3.19	0.93	0.93	8.40	11.1	0.98	2.79	7.65
	21.3	5.12	5.04		60.0	0.91	2.8	7.52
	25.8	5.82	5.73		68.2	0.93	2.07	7.55
	32.3	6.36	6.25		74.3	0.61	1.6	7.62
	58.0	7.54	7.33		87.4	1.10	2.3	7.52
4 †	9.86	1.22	1.18	8.59	13.7	48.3	39.5	7.08
	25.7	3.09	3.00		35.0	45.6	37.5	7.08
	31.35	3.67	3.56		41.4	47.2	37.3	7.06
	39.85	4.45	4.31		50.3	43.5	35.0	7.07
	68.7	5.92	5.68		66.2	42.3	33.0	7.07
	109.6	7.36	6.97		81.0	36.4	30.1	7.08
	151.	8.11	7.58		88.3	33.2	28.4	7.10
	182.	8.65	8.00		93.3	33.8	28.3	7.09
4 †	12.0	1.41	1.37	8.59	15.9	49.6	39.6	7.06
	25.1	2.76	2.67		31.1	49.7	39.2	7.06
	37.4	3.45	3.32		38.6	50.7	37.8	7.03
	54.4	4.37	4.18		48.7	49.6	37.7	7.04
	104.6	7.00	6.63		77.3	42.4	32.8	7.05
	149.5	7.22	6.69		77.9	41.3	32.6	7.06
	148.2	7.43	6.90		80.3	41.1	31.5	7.04
	228.	8.38	7.57		88.3	41.2	30.3	7.03
3	4.05	0.55	0.54	8.40	6.45	50.7	26.0	6.84
	19.8	1.52	1.45		17.3	50.0	25.7	6.84
	27.35	2.15	2.05		24.4	50.7	26.2	6.84
	59.9	3.57	3.36		40.0	50.8	24.9	6.81
	102.6	4.31	3.95		47.2	50.3	24.9	6.82
	197.5	5.99	5.29		62.8	49.6	23.3	6.80
	303.5	6.92	5.84	8.28	70.5	46.7	22.0	6.79
	593.	8.73	6.62	8.19	80.7	46.4	20.7	6.76
	732.	9.05	6.45	7.94	81.3	48.5	21.3	6.75

TABLE II—*Continued*

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
4	7.0	0.72	0.70	8.59	8.15	41.0	19.8	6.80
	12.8	1.12	1.07		12.5	41.8	20.2	6.80
	34.5	2.22	2.10		24.5	39.4	19.5	6.82
	80.4	3.87	3.58		41.7	38.8	16.6	6.74
	106.5	4.39	4.02		46.8	37.6	18.1	6.80
	150.	5.01	4.48		52.1	34.9	16.6	6.78
	250.	6.34	5.45		63.5	31.2	14.1	6.77
	743.	9.08	6.44		75.0	25.1	11.6	6.78
3	34.6	1.88	1.76	8.40	20.9	390.	70.6	6.13
	64.3	2.24	2.00	8.32	23.8	374.	67.2	6.12
	106.	2.88	2.50	8.03	31.2	404.	72.0	6.11
	150.	3.39	2.86	7.96	35.8	374.	66.6	6.11
	378.	4.35	3.01	7.24	41.6	377.	68.7	6.13

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

The curves at intermediate pH values are such that n lies between 1 and 2. The curve for the tautog at pH 7.4 is apparently a straight line of slope 1.3 so that equation (1) is applicable whereas the intermediate goosefish curves would seem to be slightly curved, indicating that a more complicated equation would give a more satisfactory description.

There is no question but that equation (2) could be used to describe these curves but their simple character renders the application of such a complicated expression of little interest.

Equation (3) may also be successfully applied here. The smooth curves in Figs. 4 and 5 are drawn according to this equation using the constants given in the legends.

Thus the dissociation curves of the tautog and the goosefish are so simple in character that they may be described by equations based on any one of the theories of the mode of combination between oxygen and hemoglobin, and in themselves give no basis for a preference of one theory over another.

These curves leave little doubt that the decrease in oxygen in acid solution is really due to a loss in ability to combine with oxygen, and not to a "Bohr effect," for certain of the curves have the same dissociation constant and differ only by the total amount of oxygen with which they are able to combine when the acidity is changed. The

TABLE III

Oxygen Dissociation of Tautog Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
6 †	9.56	1.45	1.42	9.66	14.7	12.60	56.1	7.86
	13.13	3.04	2.99		30.9	12.5	55.0	7.85
	14.23	3.07	3.02		31.3	11.80	55.1	7.88
	21.8	4.90	4.82		50.0	10.97	52.4	7.89
	38.7	7.07	6.93		71.7	10.02	50.2	7.91
	42.9	7.73	7.58		78.5	10.35	50.1	7.90
	84.2	9.09	8.79		90.7	9.59	49.1	7.92
2	12.9	1.17	1.12	8.74	12.7	9.13	15.05	7.41
	15.2	1.52	1.47		16.7	7.62	13.50	7.45
	22.6	1.97	1.89		21.5	7.89	14.35	7.46
	46.7	4.24	4.06		46.1	8.08	12.33	7.38
	70.1	5.10	4.85		55.0	7.98	12.12	7.38
	155.	7.33	6.80		77.2	5.73	9.50	7.41
	276.	8.92	7.95		90.2	5.11	8.06	7.40
4	16.4	1.38	1.32	9.39	14.2	25.8	26.1	7.18
	52.5	2.89	2.71		29.1	26.3	26.3	7.17
	109.5	4.89	4.50		47.9	19.86	22.3	7.23
	152.5	5.62	5.08		54.1	21.1	22.2	7.20
	214.5	6.45	5.69		61.1	18.8	20.8	7.22
	268.	7.08	6.13		65.9	19.3	20.5	7.20
	381.	8.37	7.02		75.5	16.8	17.8	7.20
3	13.9	1.10	1.05	7.66	13.7	118.8	36.8	6.54
	30.5	1.78	1.67		21.8	116.6	36.2	6.54
	53.8	2.30	2.11		27.5	118.5	36.5	6.54
	87.9	2.59	2.28		29.7	137.4	38.9	6.48
	124.7	3.14	2.70		35.3	130.2	36.6	6.48
	147.0	3.46	2.94		38.4	106.8	33.6	6.55
	150.8	3.36	2.83		37.0	130.7	36.8	6.48
	163.8	3.61	3.03		39.5	117.6	36.5	6.54
	309.0	4.62	3.52		45.8	123.0	36.4	6.51
	625.0	5.91	3.69		48.0	134.2	37.6	6.46
5 ‡	23.3	0.82	0.74	9.54	7.74	107.3	14.33	5.73
	39.6	1.34	1.20	8.38	14.3	104.5	15.10	5.87
	54.2	1.39	1.20	7.87	15.2	106.0	13.60	5.67
	81.9	1.55	1.26	7.77	16.2	110.8	14.49	5.71
	101.8	2.25	1.89	8.44	22.4	107.0	14.94	5.82
	155.0	2.33	1.78	8.00	22.31	109.8	14.18	5.69
	670.0	5.06	2.68	8.52	31.4	108.2	14.46	5.76

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

‡ Lactic acid added to the blood.

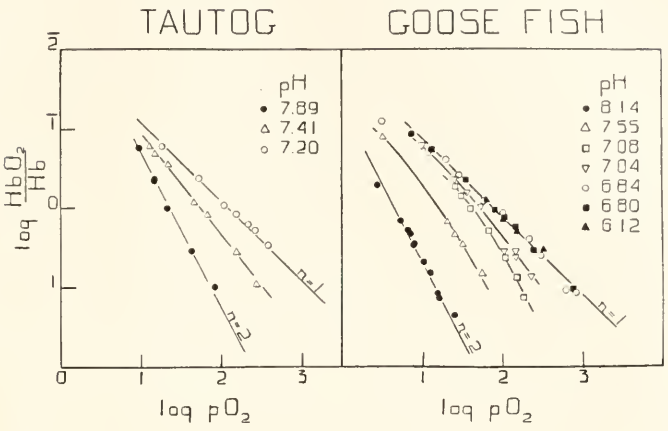

FIG. 3. For explanation of this figure, see text.

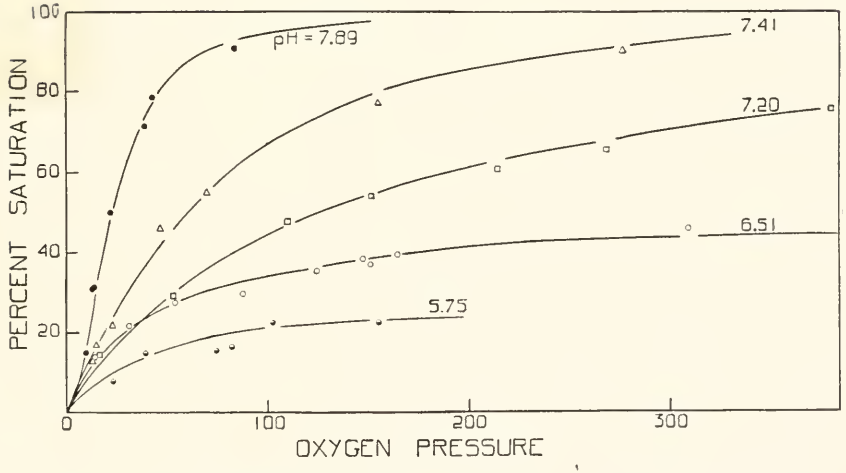

FIG. 4. Oxygen dissociation curves at constant pH of the blood of the tautog. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_0	K_1	$K_2 \times 10^2$	$1/K_1$	$1/K_2$
7.89	0	1.0	0		.19		23^2
7.41	.5	.5	0	.0286	.0156	35	80^2
7.20	1.0	0	0	.008		125	
6.51	.5	0	.5	.022		45	
5.75	.3	0	.7	.022		45	

more acid curves for tautog blood are rectangular hyperbolæ and K_1 is the same at pH 6.5 and 6.1 although the oxygen capacity is

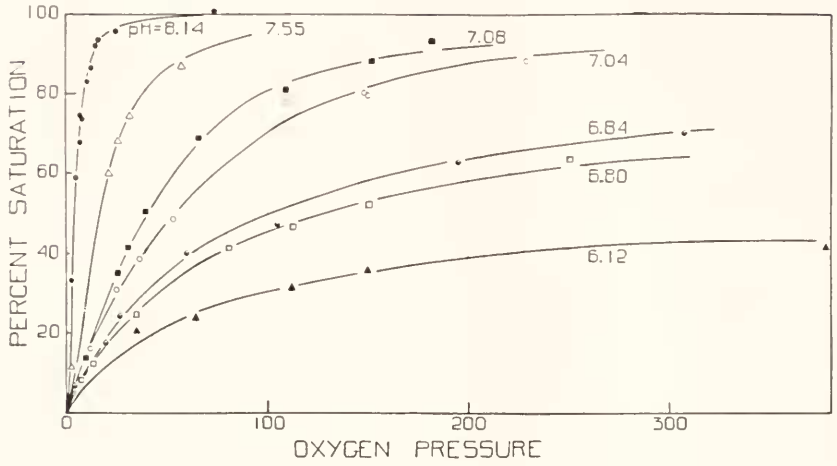

FIG. 5. Oxygen dissociation curves of the blood of the goosefish at constant pH. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_0	K_1	K_2	$1/K_1$	$1/K_2$
8.14	0	1.0	0		.05		4.47[2]
7.55	.25	.75	0	.067	.0011	15	30[2]
7.07	.45	.55	0	.04	.0004	25	50[2]
7.04	.50	.50	0	.04	.000158	25	80[2]
6.84	.88	0	.12	.0131		76	
6.80	.80	0	.20	.0131		76	
6.12	.54	0	.46	.0131		76	

diminished. The same is true of the goosefish blood at pH 6.84, 6.80, and 6.12.

The change in the position of the curves with pH is very marked in these bloods as well as in that of the toadfish. In the tautog there is an additional phenomenon. The oxygen dissociation constant for the curve at pH 7.2 is considerably smaller than that for the curves at lower pH values. That is, at first the curves are shifted to the right with increasing acidity but at higher acid concentrations there is a reversal of the effect. A similar reversal of the Bohr effect was found by Rona and Ylppö (1916) working on dog blood, by Ferry and Green (1929) for horse hemoglobin, and by Stedman and Stedman (1926) and Hogben (1926) for crustacean hemocyanin.

The most interesting single characteristic of these curves is the change in shape from a rectangular hyperbola to a sigmoid curve. Unlike the toadfish, in which one component behaves as though $n = 2$ and one component as though $n = 1$ at all pH values, the change in shape must be due to a change in behavior of the component groups.

Thus, whatever the theory used as a basis for the description of the curves, it may be observed that acidity has a threefold effect on the blood; first, a change in the value of the oxygen dissociation constant, second, a decrease in oxygen capacity, third, a change in the shape of the curves describing the equilibrium with oxygen.

Theoretical Deductions Concerning the Loss in Oxygen Capacity in Acid Solutions

Henderson (1920) has shown that the effect of acid on the combining power of hemoglobin with oxygen could be described in terms of four mass law expressions; one for the combining power of oxygen with acid hemoglobin, HHb, one for oxygen with salt hemoglobin, BHb, and one each for the acid dissociation of oxygenated and reduced hemoglobin, $HHbO_2$ and HHb respectively. Thus the ease with which oxygen combines with hemoglobin depends upon the dissociation of the latter as an acid and oxyhemoglobin is a stronger acid than reduced hemoglobin.

The situation becomes rather complicated when more than one molecule of oxygen is considered to combine with one molecule of hemoglobin. By making certain assumptions concerning the interdependence of the four prosthetic groups, it is possible to derive an expression predicting the relation between oxygen capacity and hydrogen ion concentration exhibited by the data in Fig. 1. By making a few assumptions it is possible to explain this phenomenon of reversible loss of oxygen capacity in relatively simple chemical terms. The fundamental conception is that the oxygen combination is intimately dependent upon the acid dissociation of the hemoglobin as in mammalian blood, but that the effect is so exaggerated that oxygen, at ordinary oxygen tensions, will not combine with the hemoglobin unless the latter is in the ionized state. This is, of course, not the only possible theory but it is set forth here as a simple interpretation of the mechanism of a phenomenon apparently very different from our usual conception of the equilibrium between oxygen and hemoglobin.

Let it be assumed:

1. That each hemoglobin molecule contains four groups capable of

combining with oxygen and that each of these groups is associated with the dissociation of two hydrogen ions.[2]

2. Assume further that only the ionized prosthetic groups, that is, the groups from which hydrogen ions have been dissociated, can combine with oxygen.[3] Then in the presence of oxygen sufficient to combine with all the ionized hemoglobin the number of forms present is reduced to H_8Hb, $H_6HbO_2^{=}$, $H_4Hb(O_2)_2^{\equiv}$, $H_2Hb(O_2)_3^{\equiv\equiv}$, and $Hb(O_2)_4^{\equiv\equiv}$.

3. Assume finally that the first two pairs of H ions dissociate from the hemoglobin separately but the last two pairs dissociate in a single step; then there never is present an appreciable amount of the form which can combine with three molecules of oxygen. This is in accordance with the usage of equation (3) as applied to the oxygen dissociation curves of toadfish blood.

The equilibrium reactions for the acid and oxygen dissociation of the hemoglobin molecule according to these restrictions are reduced to

$$H_8Hb = H_6Hb^{=} + 2H^+ \qquad H_6Hb^{=} + O_2 = H_6Hb(O_2)^{=}$$

$$H_6Hb(O_2)^{=} = H_4HbO_2^{==} + 2H^+ \qquad H_4HbO_2^{==} + O_2 = H_4Hb(O_2)_2^{==}$$

$$H_4Hb(O_2)_2^{==} = Hb(O_2)_2^{\equiv\equiv} + 4H^+ \qquad Hb(O_2)_2^{\equiv\equiv} + 2O_2 = Hb(O_2)_4^{\equiv\equiv}$$

If the oxygen tension is sufficiently high to convert practically all of the hemoglobin from which the hydrogen has been dissociated into the corresponding oxygenated form, the intermediate product of each pair of equations is never present in appreciable amount and the following equilibrium reactions and mass law expressions may be assumed to describe the limiting conditions:

$$H_8Hb = H_6HbO_2^{=} + 2H^+ \qquad \frac{[H_6HbO_2^{=}]}{[H_8Hb]} = \frac{K_1}{[H^+]^2}$$

$$H_6HbO_2^{=} = H_4Hb(O_2)_2^{=} + 2H^+ \qquad \frac{[H_4Hb(O_2)_2^{==}]}{[H_6HbO_2^{=}]} = \frac{K_2}{[H^+]^2}$$

$$H_4Hb(O_2)_2^{\equiv} = Hb(O_2)_4^{\equiv\equiv} + 4H^+ \qquad \frac{[Hb(O_2)_4^{\equiv\equiv}]}{[H_4Hb(O_2)_2^{==}]} = \frac{K_3K_4}{[H^+]^4}$$

[2] The assumption that the combination of each oxygen molecule is accompanied by the dissociation of two hydrogen ions is in accordance with the finding of Ferry and co-workers (1929) that K_1 in equation (2), as applied to horse hemoglobin and to sheep blood, varies with the square of the hydrogen ion concentration, and with the observation of Redfield and Ingalls (1932) that the oxygen dissociation constant for certain hemocyanins also varies with the square of the hydrogen ion concentration.

[3] This also has a parallel in the hemocyanins, where the addition of acid produces a colorless compound incapable of combining with oxygen. See the end of this discussion.

The relative concentrations of the different forms present at any given pH may be readily calculated either by the logarithmic form usually employed in treating titration curves or in an algebraic form similar to the oxygen dissociation equations. Thus for the first equilibrium if

$$[H_8Hb] + [H_6HbO_2^{=}] = 1,$$

then

$$\frac{[H_6HbO_2^{=}]}{1 - [H_6HbO_2^{=}]} = \frac{K_1}{[H^+]^2} \text{ and } [H_6HbO_2^{=}] = \frac{K_1}{[H^+]^2 + K_1}$$

and similarly for the other expressions,

$$[H_4Hb(O_2)_2^{\equiv}] = \frac{K_2}{[H^+]^2 + K_2}, \text{ and } [Hb(O_2)_4^{\equiv\equiv}] = \frac{K_3K_4}{[H^+]^4 + K_3K_4}.$$

The total amount of oxygen bound at any given pH is the sum of all of the oxygenated forms.

$$H_6HbO_2^{=} + H_4Hb(O_2)_2^{\equiv} + Hb(O_2)_4^{\equiv\equiv}$$

Since only one quarter of the total oxygen bound can be in the form $H_6HbO_2^{=}$, the concentration as derived from the equilibrium expression must be multiplied by 0.25 as must also the concentration of $H_4Hb(O_2)_2^{\equiv}$, while the concentration of $Hb(O_2)_4^{\equiv\equiv}$ must be multiplied by 0.50. Substituting these values and the mass law expressions for the various oxygenated forms the total oxygen combined, Z, becomes

$$Z = .25\frac{K_1}{[H^+]^2 + K_1} + .25\frac{K_2}{[H^+]^2 + K_2} + .50\frac{K_3K_4}{[H^+]^4 + K_3K_4}.$$

In applying these equations to the data in Fig. 1, it must be kept in mind that the shape of the oxygen dissociation curves indicates that the hemoglobin would combine with slightly more oxygen if the oxygen tension were sufficiently increased. The theoretical oxygen capacity may be calculated in the more acid range because the curves are simple rectangular hyperbolæ and in all three bloods the oxygen dissociation constant has the same value in the most acid curves. If it be assumed that the constant is the same at intervening and lower pH values, knowing the oxygen tension and the concentration of oxygenated hemoglobin, it is possible to calculate the limiting amount of oxygen with which the hemoglobin can combine at these reactions. These values have been calculated using the data given in Fig. 1 for the goosefish below pH 6.8 where $K_1 = .013$, for the tautog below pH 6.55 where $K_1 = .022$, and for the toadfish below 6.5 where

$K_1 = .047$. At higher pH values the capacities have been calculated somewhat less accurately. The points between pH 6.55 and 6.96 for the tautog have been calculated using the K_1 values at both these reactions, then averaging the results which are then $\pm$ 2 per cent of

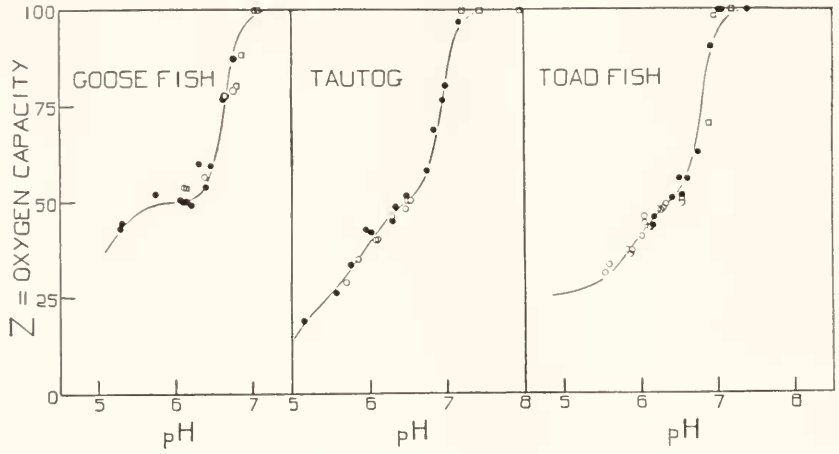

FIG. 6. The oxygen capacity of certain fish bloods. The points are calculated as described in the text:

- ● from the blood equilibrated with oxygen as given in Fig. 1.
- ○ from the blood equilibrated with air as given in Fig. 1.
- □ from the oxygen dissociation curves in Figs. 2, 4, and 5.

The curves are drawn according to equation (4).

the correct value. At oxygen tensions of 700 mm. the first half of the toadfish hemoglobin is practically saturated and the capacity between pH 6.55 and 7.0 has been calculated according to equation (3) using K_2 as 3.33×10^{-6}. All of the calculated points are represented in Fig. 6.

The curves in this figure are drawn according to equation (4) where $\sqrt{K_2}$ and $\sqrt[4]{K_3K_4}$ are for the goosefish $10^{-5.1}$ and $10^{-6.65}$ respectively; for the tautog, $10^{-5.93}$ and $10^{-6.95}$ respectively, and for the toadfish $10^{-5.85}$ and $10^{-6.8}$ respectively. $\sqrt{K_1}$ is approximately 10^{-5} for the tautog but is too large to be determined for the other two fishes.

The applicability of this type of equation to the oxygen capacity supports the interpretation of the oxygen dissociation curve as developed in the case of the toadfish. The equation is also adequate to describe the relation observed in tautog and goosefish blood, although the assumptions used in deriving curves to describe the oxygen equilibrium are somewhat different from those employed for toadfish blood. This difference suggests that in some cases there may be an

interdependence of the acid dissociation of the prosthetic groups which is not paralleled by an interdependence in the combination of oxygen.

The most important implication of the curves in Fig. 6 is the same as that derived from the undulatory character of the oxygen dissociation curves of the toadfish, namely, hemoglobin contains prosthetic groups differing in their behavior with respect to hydrogen dissociation and oxygen combination.

The evidence for this is the inflection of the tautog and toadfish curves at 50 per cent saturation and the actual break in the goosefish curve at the same point. Thus the effect of the addition of acid, as originally suggested (Root, 1931), may be interpreted as the inactivation of prosthetic groups on the hemoglobin molecule. This inactivation is probably due to the undissociated character of the hemoglobin as an acid, oxygen combining with only the ionized form of hemoglobin.

A similar inactivation of respiratory pigment in the presence of acid has been described by Redfield, Mason, and Ingalls (1932). The hemocyanin of *Limulus polyphemus* reacts with hydrochloric acid to form a component that is colorless and does not react with dissociable oxygen. The colorless component may be separated from a partially acidified hemocyanin solution by the addition of a strong solution of sodium chloride. When such a separation has been effected, analysis of the nitrogen in the filtrate indicates that its protein content has been diminished to just such an extent as the hemocyanin has been decolorized. Here, also, the combination of each oxygen molecule appears to be dependent upon the dissociation of two hydrogens, for the oxygen dissociation curves are rectangular hyperbolæ and the equilibrium between hydrochloric acid, hemocyanin, and the resulting colorless component may be described by an equation in which the hemocyanin is behaving as a divalent acid or base.

We wish to express our thanks to Professor Alfred C. Redfield for his generous interest and direction throughout the course of this investigation.

Summary

1. The oxygen dissociation curves of the blood of certain marine teleosts, the toadfish, the goosefish, and the tautog, have been studied.

2. The oxygen dissociation curves for the toadfish are undulatory in character with two areas concave to the abscissa. The second inflection begins at approximately one half saturation.

3. The oxygen dissociation curves for the goosefish and the tautog change shape with change in pH. At alkaline reactions the curves are sigmoid whereas in acid solutions they are rectangular hyperbolæ.

4. The form of the oxygen dissociation curves at constant pH can be described by Hill's equation only under a limited number of circumstances. The curves for the toadfish may be described in terms of equations derived from the assumption that each molecule of hemoglobin combines with four molecules of oxygen.

5. The position of the dissociation curves of all of these fish bloods is markedly affected by pH, and, in addition, with increasing acidity the oxygen capacity is reduced.

6. The manner in which the addition of acid lowers the oxygen capacity may be deduced from the assumption that oxygenation is dependent upon the dissociation of hemoglobin as an acid.

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